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Jameel Ahmad KhanUniversity of Agricultural
Sciences, GKVK, Bengaluru,
Karnataka, India**Baba Fakrudin**University of Horticultural
Sciences, Bagalkot, GKVK Post,
Bengaluru, Karnataka, India**Edries Yousaf Hajam**IFOM-in Stem Joint Research
Laboratory, Institute for Stem
Cell Biology and Regenerative
Medicine, Bangalore, Karnataka,
Karnataka, India**Zahoor Ahmad Dar**DARS, Budgam (SKUAST-K),
Srinagar, Jammu and Kashmir,
India**Halagappa Eshwarappa
Shashidhar**University of Agricultural
Sciences, GKVK, Bengaluru,
Karnataka, India**Corresponding Author:****Halagappa Eshwarappa
Shashidhar**University of Agricultural
Sciences, GKVK, Bengaluru,
Karnataka, India

Innovative and frugal protocols for RNA related experiments

Jameel Ahmad Khan, Baba Fakrudin, Edries Yousaf Hajam, Zahoor Ahmad Dar and Halagappa Eshwarappa Shashidhar

Abstract

Rice is staple crop and is being cultivated far and wide around the globe. Waterlogged habit of cultivation has made rice extremely sensitive to drought. Understanding expression profiles will open new avenues of research for drought tolerance in rice. Present study aims at utilizing the most innovative and frugal ways for highly sensitive molecular biology protocols. These protocols will help researchers to undertake RNA work in any lab with basic amenities. A details methodologies with critical tips is compiled and presented here for RNA extraction, RNA detection and finally expression profiles of two genotypes. This proof of concept for novel methods is a complete guide for beginner in RNA work with minute detailing of each step.

Keywords: Bleach protocol, Drought, Primerdimer, Rice, RNA, RNase, qPCR

Introduction

Water is an important factor for crop production and its shortage has created negative impact on the food security of the world. Rice (*Oryza sativa*) is one of the important cereal crops and serves as the staple diet for a large number of people on earth. Cultivation of rice is effected by various abiotic stresses compromising the physiological processes and decrease quantity and quality (Wang and Frei 2011; Shashidhar *et al.*, 2013) [12].

Rice is sensitive to drought and scientists have been working to increase the water use efficiency of crops through breeding or genetic transformations (Varma *et al.*, 2012; Babu *et al.*, 2016) [14, 1]. Many drought resistant genotypes have been produced and drought resistance genes have come into picture (Shashidhar, 2007). Many studies have been performed in rice to improve quality traits (J. A. Khan *et al.* 2018) [9] and yield associated traits by identifying stress responsive genes (Wang *et al.*, 2015; Steele *et al.*, 2006) [15, 13]. However, expression profiling on the role of drought response and tolerance related genes has been lagging particularly new phenotyping technologies need to be combined with functional validations. Understanding expression profiles will help in dissecting drought tolerance mechanisms and ultimately providing practical solution to drought problem.

Polyethylene glycol (PEG) is a water soluble compound which does not change pH of solution. PEG is non-toxic polymer and is the most widely used to create osmotic stress which mimics decreases in soil water potential. PEG solutions is used to simulate drought conditions in short-term experiments, which would be useful for evaluating the suitability of new sources of germplasm, and also for screening of large populations from the early generations in breeding programs.

As a good model plant, rice is relatively easy to transform and is suitable for various molecular, biochemical, genomics and other interventions. Isolation of high-quality ribonucleic acid (RNA) from various rice materials and Quantitative Real Time PCR (qPCR) is a prerequisite for the abovementioned studies. Total RNA is also useful for RNA-seq, cDNA library construction, microarray studies, northern blot analysis etc.

Quantitative reverse transcription PCR (RT-qPCR) is a widely used for transcript analyses. Results of qPCR are authentic molecular biology technique for the validation of gene expression. In this technique, cDNA is usually used as template converted from RNA which represents instant transcript stage of organism. Fluorescent tag molecules are used to analyse real time synthesis of amplicons. The products formed are monitored in digital information at each cycle (Gachon *et al.*, 2004) [5]

In the present study we provide a holistic method from RNA isolation, cDNA synthesis and qPCR. This protocol contains many modifications and troubleshoots at every step for quick, easy and efficient process. Starting with modified conventional TRIzol reagent method, bleach method for RNA isolation, TAE (Tris base, Acetic acid and EDTA) method for determining RNA quality and elimination of primer dimers are key concerns addresses in this study which can be applied in any low to medium scale laboratory.

The following protocol describes the total RNA extraction from rice seedlings and young tissues/organs using TRIzol reagent, which is an improvement to the single-step RNA isolation method developed by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987) [3] followed previously in other rice study (Yin *et al.* 2016) [16] with additional information of RNase management.

I. Sample preparation

Plant material

Rice (*Oryza sativa* L.) seeds (100-200) of two varieties were taken for the study. Initially, surface sterilization of rice seeds was done with MiliQ water followed by 70% ethanol for 1 min. Sodium hypochlorite (HiMedia) was used with its 4% v/v concentration and finally seeds were washed with distilled water (Fig. 1). Surface sterilized seeds were allowed to pre-germinate in growth room (designated as *Arabidopsis* growth chamber).

After surface sterilization keep the seeds in closed oakridge tube, this will help in pre germination and prevent contamination

Media preparation

1. To mimic the drought condition, Polyethylene Glycol (PEG) at 5% was used additionally in Stress/Treated experiment. In control condition, media constituents consists of ½ strength of Murashige and Skoog medium (MS media) and 3% gelrite to give transparent and gel strength.
2. MS media was added initially followed by PEG in treatment, mixing was done in orbital shaker till all the constituents are dissolved. Using pH meter pH was set to 5.7, gelrite was added at last.
3. Media prepared (both control and treated) were autoclaved and media bottles were kept at room temperature for half an hour to let it cool. 40ml of media was poured in each test-tube in Laminar Air Flow (LAF). Similar protocol was followed previously by us (Khan and Shashidhar, 2018) [8].

pH plays an important role in solidifying media, acidic pH usually prevents solidifying.

Media should be transferred to test tubes two days before the transfer of seeds and kept in LAF, consequently contaminated test tubes can be removed in advance.

Transfer of seeds

Seeds were transferred to test tubes (20x2.5cm) containing the solidified media. Each genotype was replicated 15 times for control and treated condition (Fig. 1e).

After seeds are transferred, test tubes should be covered (either with test tube caps or Parafilm). Half dipped seeds in media respond well and establish better roots.

Growth Conditions

Test tubes laid on stands were covered with opaque cover from bottom. Test tubes were kept in dark for two days in growth chamber. After the plumule/white tip (2 to 3mm) emerged, 16-hr light/day was maintained at 25-30 °C for ten days.

Contaminated test tubes should be removed as soon as they appear.

II. RNA extraction

RNases are ubiquitous and is secreted as a defensive component from skin (Harder and Schröder, 2002) [6] and other fluids produced by human body. Hence, it produces an immediate threat to the RNA extraction process. Moreover, RNases resist to wide number of chemicals, pH and temperature (Kalnitsky and Resnick, 1959; Dalaly *et al.*, 1980) [7, 4]. Normally in most of labs RNaseZAP works efficiently in removing RNase traces, but it is unaffordable to many labs. Hence, for quick and cheap RNA extraction, our laboratory replaced RNaseZAP with the use of common house hold bleach (6% sodium hypochlorite) to destroy RNases.

RNase free working place

1. Working bench was cleaned thoroughly with 70% ethanol till the liquid dries off. A working solution of 20% concentration was used to remove the RNase activity in an around the working place (Fig. 2a). No DEPC treatment is required if 20% bleach spray is done on glove.

Homogenizing tissue

1. 300-400 mg of roots from the rice seedlings were used for RNA extraction. Seedlings were pulled carefully from the test tubes and quickly wiped with tissue paper to remove any media (Fig. 2b). Roots were cut from shoots with surgical blade and immediately dipped into liquid nitrogen. Pressing of roots in between tissue paper helps in easy removal of seeds. Pull off the roots from samples where RNA extraction follows immediately. Samples stored in freezer (-80°C) should be grinded immediately without thawing.
2. Rice roots were grind, in prechilled mortar and pestle containing liquid nitrogen, into fine powder. Using prechilled spatula, this powder was transferred into 2ml RNase free tubes. Use Latex gloves sprayed with 20% bleach during entire process instead of RNaseZAP. Prechilled mortar-pestle, spatula and tubes should be used.

Total RNA extraction

1. 1ml of TRIzol reagent was added to each tube containing powdered sample and mixed vigorously for 1 min. Homogenised sample were kept at room temperature for 5-10 min with intermittent mixing to completely dissociate nucleoprotein complex (Fig. 2d). *Mixing should be done by inverting tubes several times, vortexing may lead to RNA contamination.*
2. 200µl of chloroform was added to each tube containing TRIzol-sample mixture. Vigorous shaking was given for

10 sec and incubated at room temperature until a clear distinction of aqueous and organic layer was visible.

3. Tubes were centrifuged at 12,000xg for 15 min, 4°C. (Fig. 2e) For clear distinction of aqueous and organic layers, tubes should not be disturbed for 5-10 min. Precool the centrifuge before use. After centrifuge, three layers are visible. RNA is present in upper aqueous phase whereas interphase contains DNA and proteins.
4. Among the three layers present after centrifuge, aqueous layer was pipetted out into 1.5ml RNase free tube. Tubes containing three layers should be handled carefully. Extreme care should be done to pipette out only the aqueous phase, it is recommended to leave some aqueous phase near to interphase layer. Use of 200 µl pipette for better control, usually 400-500 µl of aqueous layer is taken out. Tips should be changed for every sample. Do not take out more than six samples from centrifuge at ones. *To improve RNA quality Chloroform step can be repeated in older samples.*
5. Equal volume of isopropyl alcohol was added to the tube and mixed by inverting tube for 10sec.
6. Incubate at room temperature for 10 min and centrifuge at 12,000xg, 4 °C. RNA will be visible in the form of pellet after this step. Using marker pen, pellet could be marked for easy identification in later step.

III. Washing and dissolving of RNA

1. Supernatant was removed and RNA pellet was washed with 1ml of 75% alcohol. Tubes were gently inverted few times to completely wash the pellet. Air dry the RNA by keeping the cap open until ethanol evaporates. Dry heat at 60°C for maximum of 5 min quickens the drying process. RNA washing can be done 2-3 times to improve RNA quality. A short spin must be done after every wash. Before removing the alcohol supernatant it is recommended to give a short spin especially when RNA pellet detaches from the bottom. Do not over dry the RNA. Over dry RNA does not dissolve and needs extra step for dissolving.
2. RNA was dissolved in 50µl of DEPC or RNase free water. Over dry RNA can be diluted by heating the tube containing DEPC water in water bath at 60 °C for 5 min

IV. RNA quality

1. Using Nanodrop concentration of RNA was measured by using 1µl of sample at A_{260/280} OD Ratio of A₂₆₀ and A₂₈₀ between 1.8 to 2.1 is considered to be good quality of RNA. If RNA concentration is more than 1000ng more dilution can be done.

V. RNA integrity

1. RNA analysis with denaturing gel electrophoresis need many chemicals which are costly, time intensive and toxic like formaldehyde, formamide, or urea, or some other compounds like glyoxal/DMSO, mercuric hydroxide, guanidine thiocyanate, and SDS. The reason for using such chemical is to prevent the RNase activity and disrupt the secondary structure for separation during electrophoresis. We used TAE (Tris base, Acetic acid and EDTA) based electrophoresis for RNA analysis in the same setup of DNA analysis. This protocol has been successfully used by Lajoie and Jorcyk, 2012^[10]. They removed RNase contamination by using common

household bleach (6% sodium hypochlorite) in TAE agarose mixture prior to heating. We found adding of bleach (1ml/100ml of solution) prior or after heating had similar effects. Also, we used DEPC water to prepare TAE stock and working solution. We found both of them gave convincing results by oxidising the RNase and other degrading proteins. Also secondary structures were resolved and proper separations of bands were seen after gel documentation (Fig. 3).

Integrity of RNA was checked using the above mentioned bleach protocol. Earlier non-denaturing agarose gel electrophoresis was used which is time consuming and requires many toxic costly chemicals.

2. Agarose gel was prepared using 1xTAE using 2% Agarose. 1ml of household bleach containing 6% sodium hypochlorite was added before or after heating of agarose mixture. The solution was incubated at room temperature for 5-10 min if bleach was added before heating or 10-15 min if bleach was added after heating of agarose solution. It is preferable to use DEPC treated water to prepare stock and working solution of TAE, Ethidium bromide and loading dye. However, MiliQ water which is RNase free also works for the protocol.
3. 6 µl of Ethidium bromide (5mg/ml) was mixed to 100ml of agarose solution and poured into molds containing combs to solidify.
4. 1µl of RNA sample was mixed with 1 µl of 2x loading dye and loaded into wells. The gel was run for 30 min with constant voltage (CV) of 100V. Gel was imaged using gel documentation unit (Fig. 3) Prevent instrument to go into Constant Current (CC) as it may cause overheating in buffer which caused RNA degradation. It is essential to use DNase treatment separately or in combination with cDNA synthesis. Even though we could not find gDNA contamination in samples (no gDNA specific band in figure), we still followed the gDNA wipe-out protocol as precaution using Qiagen cDNA synthesis kit.

VI. cDNA synthesis

1. Single stranded cDNA was prepared by using High Capacity cDNA Reverse Transcription kit (cat#205311, QuantiTect®, Qiagen®) as per the manufacturer's protocol. All reagents were thawed and stored on ice in RNase and DNase free work environment. All individual reagents were mixed thoroughly and spin down and pipetted. About 1 µg of total RNA in a single 14 µl reaction to remove genomic DNA as mentioned in Table 1. The reaction was incubated in water bath for 2min at 42 °C, and then placed immediately on ice.
2. Reverse Transcription Master Mix was prepared as per Table 2. RNA from previous step was added to make 20 µl reaction which quantitatively converted to single-stranded cDNA using standard thermal condition mentioned in Table 3.

VII. Standardisation of Primers

1. The primer pairs for predicted genes were designed online on Integrated DNA Technology (IDT) using Prime Quest tool (<https://eu.idtdna.com/Primerquest/Home/Index>) and the primers were synthesized at Juniper Life Sciences, Bangalore. A predicted melting temperature (*T_m*) of 60+ 2 °C, primer lengths of 20-24 nucleotides, guanine-cytosine (GC) contents of 45-55 per cent and PCR

amplicon length of 100-200 base pairs (bp) were adapted for designing the primer pair. The primers were further checked for Hairpin loop formation/secondary structure/duplex formation using inbuilt feature of PrimeQuest. Primer design on exon-exon junction prevents false positives due to gDNA contamination. The specificity of the newly designed primers can be checked using NCBI Megablast.

- Primer concentration and annealing temperature are important parameters to attain specific and reproducible results. Using stock solution having 100pmol/ μ l concentration, different working solution having 2, 3, 4 and 5 pmol/ μ l were prepared. Primer pair was titrated for optimal annealing temperature and divided into three group ranges viz. 55-65 °C. Primer at 2pmol/ μ l concentration gave specific visible band and was used for downstream process. However, still primer dimers (PD) could be observed in melting and amplification curve (Fig. 4). We troubleshoot this problem by modifying the qPCR protocol. Normally primers are to be resynthesized if primer dimer persists in gradient PCR, but with the modified qPCR conditions single fluorescence peak was obtained. PDs exhibit a lower T_m than the desired amplicon as they are considerably shorter. Taking advantage of this knowledge and the ability of machine to read fluorescence at any point, PD interference was eliminated. This was done by adding an additional step having temperature one degree higher than PD melting temperature. This protocol was followed previously by (Ball *et al.*, 2003) [2] Machine was set to quench fluorescence at this stage (Fig. 5), all the PD would be single stranded and fluorescence of only desired amplicon is measured.

VIII. qPCR

- Applied Biosystem StepOne™ Real-Time PCR (California, USA) was used for all real-time PCR amplifications. The optimal PCR conditions used for amplification are given in Table 4.
- In order to analyze the gene expression by using the 2- $\Delta\Delta C_q$ method described by (Livak and Schmittgen 2001) [11], a validation curve was generated to check efficiency of machine, and the slope was used to determine suitability of the designed primers for the analysis (Fig. 6).

Materials and Methods

I. Surface Sterilization

Ethanol (70%)
Sodium Hypochlorite
Polyethylene Glycol (PEG)
Murashige and Skoog medium (MS media) media (without hormones and vitamins)
Gelrite
Mili Q water
Test tubes (25x200mm) and test tube stands
pH meter
Parafilm
Autoclave and Laminar Air Flow (LAF)

II. Plant Material for RNA extraction

To explain the protocol only the data of two rice genotypes (ARB6 and Budha) are presented here. ARB6 is represented as 01 and Budha as 02 in figures. Both the genotypes were having two treatments designated as Control (C) and Stress

(S). Control treatment contains only 1/2 strength MS media in 3% Gelrite, Stress treatment has additionally PEG @ 5% to induce osmotic stress. Each treatment was replicated 15 times and was kept in same conditions of light and temperature for 10 days. All the biological replicates of particular treatment were combined for RNA extraction.

Extra five replicates (i.e., 20 replications) were prepared for each treatment. Only healthy and uniform 15 replicates must be used for RNA extraction.

Primers were designed on OsARF gene and was used for expression analysis for the two genotypes under two treatments

III. Preparation of RNase free labware

Diethyl pyrocarbonate (DEPC)
House hold bleach (6% hypochlorite)
Mortar and Pestle
Spatula
Beaker
Pipette and tips
1.5-ml and 2-ml microcentrifuge tube
Aluminum foil
Autoclave

Common house hold bleach containing 6% sodium hypochlorite was used as stock. All the materials except RNase free tubes and tips were washed with 20% of bleach and then with RNase free MiliQ water. Mortar, Pestle, Spatula and pipette were covered with Aluminium foil packed in autoclavable cover.

Gloves sprayed with 20% bleach should be used during the whole process

IV. RNA Isolation

TRIzol
Cholorform
Isopropyl alcohol/ Isopropanol
Ethanol (75%)
DEPC water
To prepare DEPC water, add 1ml of DEPC to 999ml of water in a brown bottle. Mix overnight in an orbital shaker at 37°C and autoclave.

V. cDNA synthesis

cDNA synthesis kit from Qiagen to be used as per user guidelines

VI. qPCR

qPCR strips
SYBR green
qPCR Machine (Applied Biosci)
Master-mix was prepared on ice cooler and short spin was given to strips at the end of preparation.

Cq/Ct values

Analysis of relative gene expression using real time qPCR was done using 2- $\Delta\Delta C_q$ method developed by Livak and Schmittgen, 2001 [11].

Table 1: Genomic DNA elimination reaction components

Component	Volume/reaction
gDNA Wipeout Buffer	2 μ l
Template RNA, up to 1 μ g*	Variable
RNase-free water	Variable
Total reaction volume	14 μ l

Table 2: Reverse-transcription reaction components

Components	Volume/reaction
Reverse-transcription master mix	
Quantiscript Reverse Transcriptase*	1 μ l
Quantiscript RT buffer**	4 μ l
RT primer Mix	1 μ l
Template RNA (Entire genomic DNA elimination reaction)	14 μ l
Total reaction volume	20 μ l

Table 3: Thermal condition for single-stranded cDNA conversion

	Step 1	Step 2	Step 4
Temperature	42 $^{\circ}$ C	95 $^{\circ}$ C	4 $^{\circ}$ C
Time	15 min	3 min	hold

The protocol is applicable with 10pg to 1 μ g RNA. If using >1 μ g RNA, scale up the reactions linearly

Table 4: Thermal condition for qPCR

Stage	Step	Temperature($^{\circ}$ C)	Duration (min. & sec.)	No. of cycles
1	Initial denaturation	95 $^{\circ}$ C	10 min	1
2	Denaturation	95 $^{\circ}$ C	5 sec	
3	Annealing /Extension	60 $^{\circ}$ C	20 sec	35
4	Additional Step	75 $^{\circ}$ C	20 sec.	
5	Melting curve	95 $^{\circ}$ C-60 $^{\circ}$ C	0.1 $^{\circ}$ C/min	

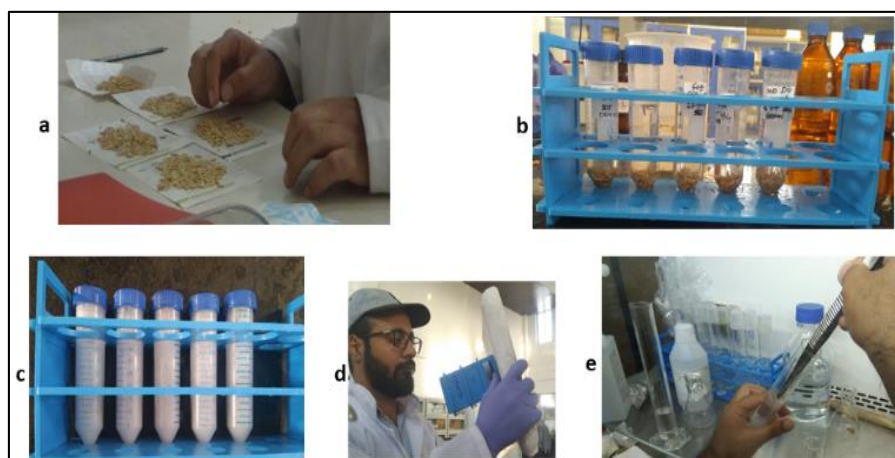


Fig 1: Critical steps of surface sterilization used in both phenomics and genomics study. a: Rice (*Oryza sativa*) seeds cleaned manually on clean tissue paper; b: Initial cleaning was done with MiliQ water, ethanol and sodium hypochloride; c: Froth appearance after adding Tween20 d: Shaking of tubes at every step and e: Transfer of seeds into test tubes containing media.

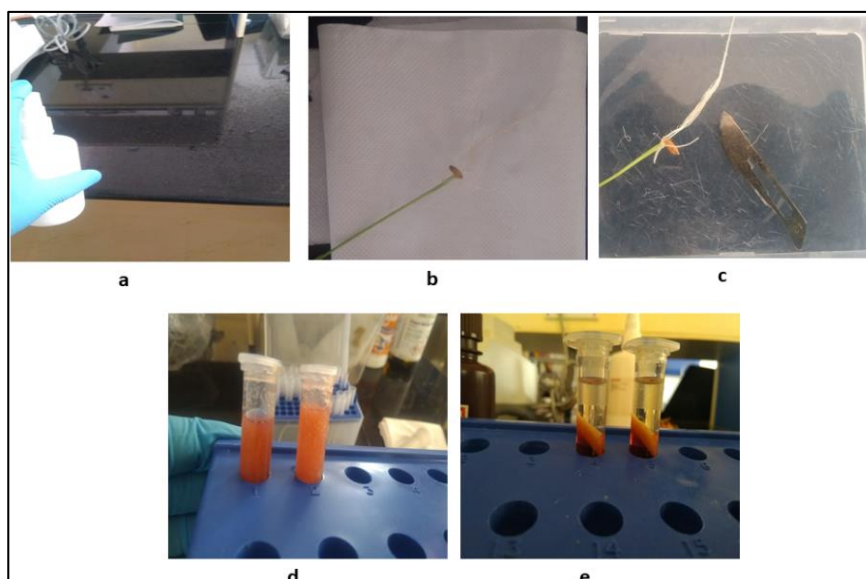


Fig 2: Critical steps in RNA extraction for expression profiling at UHS, GKVK campus. a. 20% bleach spray on working bench; b. Wiping of roots with tissue paper to remove media; c. Roots cut from shoots with surgical blade; d. TRIzol added to powdered roots and e. Typical three layers appear after spin

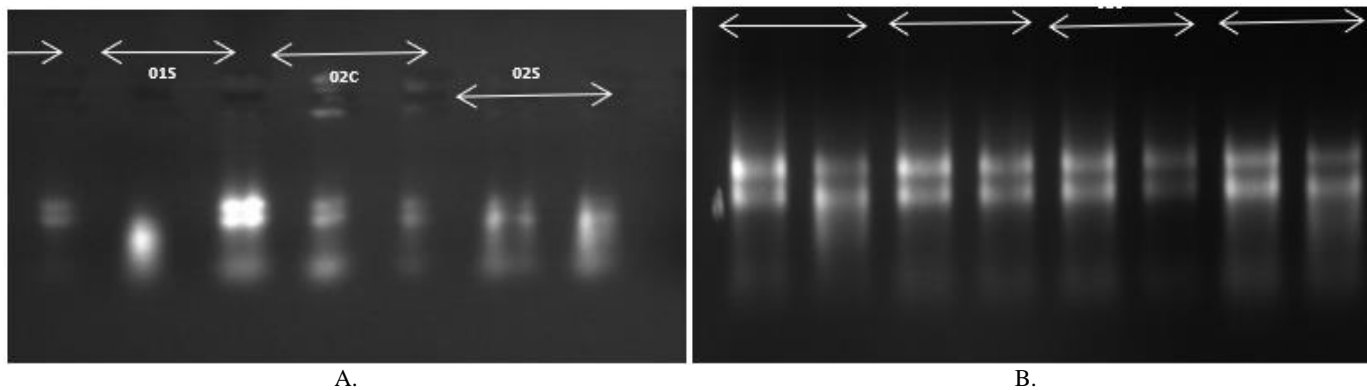


Fig 3: Confirmation of RNA integrity in two replications for each treatment on Agarose gel in TAE buffer without bleach (a) and with bleach (b). 01C-‘ARB6’ under control condition 01S-‘ARB6’ under stress condition 02C-‘Budha’ under control condition 02S-‘Budha’ under stress condition



Fig 4: Real time melt curve and amplification plot a, b) primer dimer formation in melting curve and ammonification plot respectively and c) Single amplification plot after modification in qPCR protocol showing increasing SYBR green fluoresce (ΔR_n) with PCR cycle.

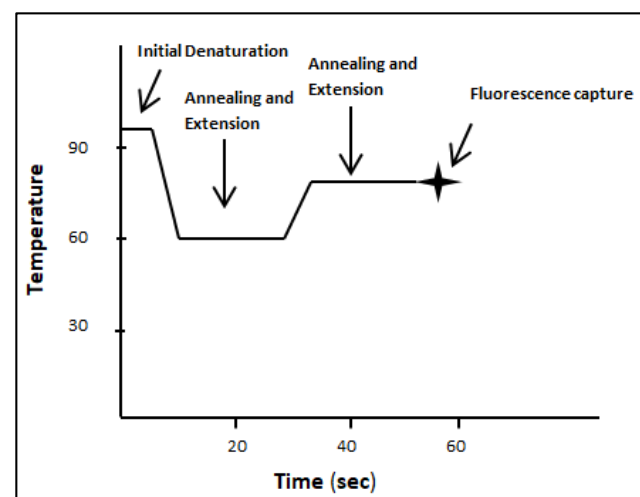


Fig 5: Modified qPCR protocol to denature primer dimer having elevated temperature during fluorescence capture followed for Cq/Ct determination

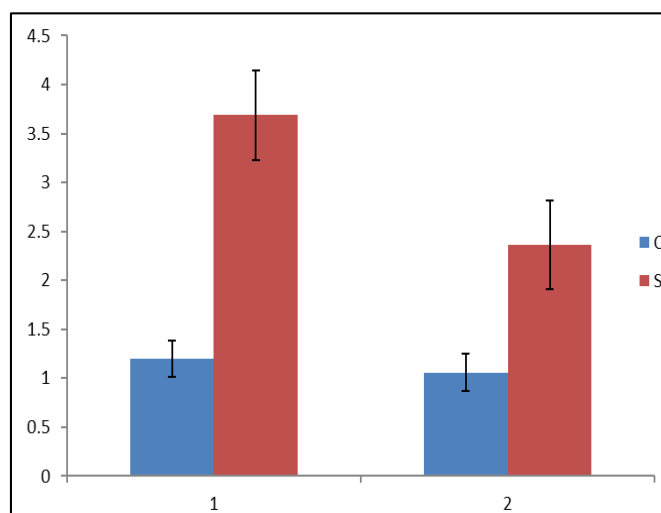


Fig 6: Expression of drought responsive OsARB gene in two rice genotypes as influenced by osmotic stress.

Conclusion

RNase are ubiquitously found everywhere and makes RNA extraction is quiet tricky. Expensive chemical and lengthy protocol are needed to eradicate the RNase contamination. In the present methodology paper, we have compiled and verified previous methods and added few more to make RNA work easy and affordable. Bleach protocol has been developed quiet early and similarly reducing primer dimers using new qPCR protocol. However, we replaced bleach with RNaseZAP and used it to spray for cleaning workbench and the gloves. We also described troubleshoots at every step. We strongly believe science will grow more if such experiential hacks are shared with research community.

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